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Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels

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Immunoglobulin A (IgA) nephropathy is the most prevalent form of glomerulonephritis worldwide. A renal biopsy is required for an accurate diagnosis, as no convenient biomarker is currently available. We developed a serological test based upon the observation that this nephropathy is characterized by undergalactosylated IgA1 in the circulation and in mesangial immune deposits. In the absence of galactose, the terminal saccharide of *O*-linked chains in the hinge region of IgA1 is terminal or sialylated *N*-acetylgalactosamine. A lectin from *Helix aspersa*, recognizing *N*-acetylgalactosamine, was used to develop an enzyme-linked immunosorbent assay that measures galactose-deficient IgA1 in serum. The median serum lectin-binding IgA1 level was significantly higher for 153 Caucasian adult patients with IgA nephropathy without progression to end-stage renal disease as compared with that for 150 healthy Caucasian adult controls. As the lectin-binding IgA1 levels for the controls were not normally distributed, the 90th percentile was used for determination of significant elevation. Using a value of 1076 U/ml as the upper limit of normal, 117 of the 153 patients with IgA nephropathy had an elevated serum lectin-binding IgA1 level. The sensitivity as a diagnostic test was 76.5%, with specificity 94%; the positive predictive value was 88.6% and the negative predictive value was 78.9%. We conclude that this lectin-binding assay may have potential as a noninvasive diagnostic test for IgA nephropathy.

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Since its initial description in 1968,[1] immunoglobulin A (IgA) nephropathy (IgAN) has become recognized as the most common primary glomerulonephritis worldwide.[2] IgAN is characterized by mesangial deposits of IgA1 with co-deposits of C3, and often also IgG or IgM or both.[1,3–6] Over 50% of IgAN patients exhibit increased serum levels of IgA and IgA-containing circulating immune complexes (CIC).[7–9] The mesangial immune deposits likely originate from these CIC. We have found that these complexes contain aberrantly glycosylated polymeric IgA1 (galactose (Gal)-deficient in *O*-linked glycans).[10–13] *In vitro* studies have demonstrated that these CIC readily bind to mesangial cells and induce their proliferation.[12–16] Thus, aberrantly glycosylated polymeric IgA1 may be involved in the pathogenesis of the glomerular injury in IgAN. Indeed, elution of glomerular immune deposits from renal tissue of patients with IgAN has demonstrated substantial quantities of Gal-deficient IgA1.[17,18]

Several laboratories have localized the aberrant glycosylation to the *O*-linked glycans in the hinge region of some IgA1 molecules.[5,15,19–21] In the absence of Gal, the terminal *N*-acetylgalactosamine (GalNAc) residues, sialylated GalNAc, or the hinge-region glycopeptides are recognized by naturally occurring IgG or IgA1 antibodies with anti-glycan or anti-hinge region peptide specificities, thus generating CIC.[10] Some of the resultant CIC escape normal clearance and deposit in the mesangium. These observations support the postulate that *O*-glycosylation abnormalities of IgA1 predispose to mesangial IgA deposition.[5,12,14,15,21,22]

For the diagnosis of IgAN, a renal biopsy is required because there is no established serological marker. To avoid this invasive procedure, particularly in individuals with mild disease, we sought to develop a noninvasive test to support the diagnosis of IgAN using a GalNAc-specific lectin. Based on previous work by our group and others, the lectin from *Helix aspersa* (HAA) is well suited for detecting Gal-deficient IgA1 *O*-linked glycans.[10,11,15–17,23–26] In this study, we show that a quantitative lectin-based enzyme-linked immunosorbent assay (ELISA) can be successfully used to measure serum levels of Gal-deficient IgA in sera of patients with IgAN.

RESULTS

Serum IgA concentration

Median serum IgA concentration was 3 943 μg/ml (range 1 171–11 713 μg/ml) for 153 Caucasian IgAN patients who had not progressed to end-stage renal disease as compared with 2 843 μg/ml (range 886–8 185 μg/ml) for 150 healthy controls ($P<0.0001$) (Table 1). Fifty patients had a serum IgA concentration higher than 4 761 μg/ml (90th percentile for controls). These data indicate a sensitivity of 32.7% and specificity of 90% with a positive predictive value of 76.9% and a negative predictive value of 56.7% (Table 2). In agreement with a previous publication,[22] the diagnostic utility of total levels of serum IgA was not satisfactory.

Serum HAA-IgA levels

Serum HAA-IgA levels for the patient and healthy control groups are shown in Figure 1a. The individual levels for the control group were not distributed normally with a marked skewing towards elevated levels. The median serum HAA-IgA level for the patients of 1 731 U/ml (range 320–8 317 U/ml) was significantly higher than the median of 615 U/ml (range 264–1 807 U/ml) for the control group ($P<0.0001$). The serum HAA-IgA level was higher than 1 076 U/ml (90th percentile for controls) for 117 of the 153 patients. These data indicate a sensitivity of 76.5% and a specificity of 90% with a positive predictive value of 88.6% and a negative predictive value of 78.9%. To illustrate the potential of this assay, the results are also presented as a receiver operating characteristic curve in Figure 1b. The area under the curve is 0.9021 with a standard error 0.0178, indicating that the true-positive rate was high and the false-positive rate was low. The 95% confidence interval was 0.8672–0.9370 ($P<0.0001$).

Reproducibility of the HAA-IgA ELISA was high (repeated the assays had an $r=0.970$; $P<0.0001$). The serum HAA-IgA and IgA levels correlated significantly in IgAN patients ($r^2=0.571$, $P<0.0001$) and healthy controls ($r^2=0.392$, $P<0.0001$).

The median value of the HAA-binding fraction of IgA was 445 U/mg IgA (range 129–879) for 153 Caucasian IgAN patients who had not progressed to end-stage renal disease as compared with 248 U/mg IgA (range 56–666) for 150 healthy controls ($P<0.0001$). Ninety-four patients had a HAA-binding fraction of IgA higher than 394 U/mg IgA (90th percentile for controls). These data indicate a sensitivity of 61.4% and specificity of 90% with a positive predictive value of 86.2% and a negative predictive value of 69.6%. Seven of 36 patients with normal HAA-IgA levels had an HAA-binding fraction of IgA above the 90th percentile for controls; among the 135 healthy controls with a HAA-IgA level below

Table 1 | Clinical features and levels of serum IgA and HAA-IgA for subgroups of patients with IgA nephropathy

Group	N	Gender ratio (M/F)	Age at diagnosis (years) mean ± SD	Interval to study (years) mean ± SD	HAA-IgA (U/ml) median (range)	IgA (μg/ml) median (range)	HAA-IgA (U/mg IgA) median (range)	UP/Cr ratio median (range)	GFR[a] (ml/min/ 1.73 m^2) mean ± SD
All patients	153	1.68	37.0 ± 14.5	4.9 ± 6.9	1 731 (320–8 317)	3 943 (1 171–11 713)	445 (129–879)	0.60 (0.06–19.3)	57.0 ± 31.4
Male patients	96		39.8 ± 15.0	3.9 ± 5.6	1 731 (373–8 317)	3 846 (1 171–11 713)	455 (129–834)	0.65 (0.06–5.35)	53.3 ± 30.8
Female patients	57		32.2 ± 27.8	6.5 ± 8.4	1 707 (320–4 366)	4 213 (1 409–7 283)	428 (129–879)	0.54 (0.06–19.3)	63.1 ± 32.0
<8 weeks after biopsy	34	1.62	37.5 ± 14.1	0.06 ± 0.04	1 800 (508–4 366)	3 956 (1 418–6 327)	451 (160–879)	0.81 (0.07–4.36)	61.0 ± 39.0
>5 years after biopsy	46	1.09	30.2 ± 11.9	13.3 ± 7.0	1 974 (504–4 391)	4 440 (2 037–9 148)	425 (129–812)	0.19 (0.06–19.3)	60.7 ± 38.5
GFR < 30 ml/min/1.73 m^2	35	3.38	42.0 ± 15.5	4.5 ± 7.2	1 856 (391–4 391)	3 584 (1 418–8 887)	525 (141–879)	1.25 (0.07–7.46)	17.9 ± 6.8
GFR > 90 ml/min/1.73 m^2	26	1.17	27.5 ± 9.5	3.2 ± 4.9	1 466 (320–3 579)	3 299 (1 409–7 422)	373 (170–710)	0.38 (0.06–2.53)	105.0 ± 16.3
Normal urinalysis	26	1.36	30.0 ± 10.3	11.8 ± 8.9	2 030 (504–3 901)	4 571 (2 037–7 350)	417 (208–820)	0.09 (0.06–0.19)	45.5 ± 16.2

GFR, glomerular filtration rate; HAA, lectin from *Helix aspersa*; IgA, immunoglobulin A; UP/Cr ratio, urinary protein/creatinine ratio.
[a]Estimated GFR was calculated using the MDRD formula (53).

Table 2 | Diagnostic utility of serum IgA and HAA-IgA assays

	Control		Patients					
	Yes	No	Yes	No	Sensitivity	Specificity	Positive predictive	Negative predictive
IgA >4 761 μg/ml	15	135	50	103	0.327 (0.258, 0.405)	0.900 (0.842, 0.938)	0.769 (0.654, 0.855)	0.567 (0.504, 0.629)
HAA-IgA >1 076 U/ml	15	135	116	37	0.758 (0.685, 0.819)	0.900 (0.842, 0.938)	0.885 (0.820, 0.929)	0.785 (0.718, 0.840)
HAA-IgA >394 U/mg IgA	15	135	94	59	0.614 (0.535, 0.685)	0.900 (0.842, 0.938)	0.785 (0.628, 0.756)	0.696 (0.628, 0.756)

HAA, lectin from *Helix aspersa*; IgA, immunoglobulin A.
The numbers in the brackets are the 95% confidence intervals.

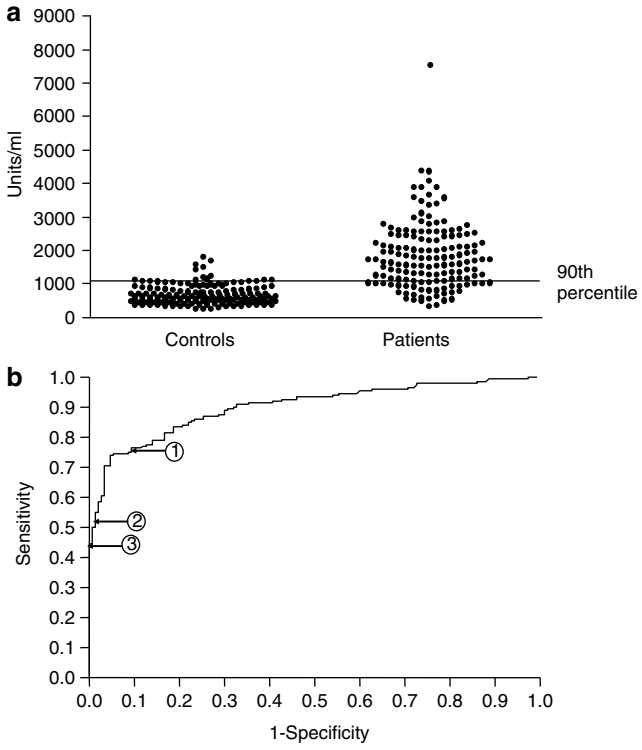

Figure 1 | Serum levels of Gal-deficient IgA in IgAN patients and healthy controls. (**a**) HAA-IgA level (U/ml, see Materials and methods) in sera from IgAN patients and healthy controls. (**b**) Receiver operating characteristic curve for HAA-IgA levels. Area under the curve is 0.902. The arrows with numbers indicate key points: (1) HAA-IgA serum level 1 076 U/ml; sensitivity 0.765, specificity 0.900; (2) HAA-IgA serum level 1 702 U/ml; sensitivity 0.503, specificity 0.993; and (3) HAA-IgA serum level 1 810 U/ml; sensitivity 0.444, specificity 1.000.

the cutoff point, 11 had a similarly increased value for HAA-binding fraction of IgA.

Western blot analysis

To verify the ELISA data, selected serum samples from four IgAN patients and two healthy controls representing a wide range of HAA-IgA values were analyzed by Western blotting for total and Gal-deficient IgA (see Materials and methods). The band intensity was measured by densitometry and the ratio of HAA-IgA to IgA heavy chain was calculated. This value was plotted against HAA-binding fraction of IgA1 (U/mg IgA) determined by ELISA in the same sample. The data in Figure 2 are representative for the IgAN patients and healthy controls over the wide range of HAA-IgA values. The results from the two assays showed good correlation ($r^2 = 0.818$, $P = 0.046$).

Clinical and pathological associations

Serum HAA-IgA and IgA concentrations as well as values for the HAA-binding fraction of IgA (U/mg IgA) are shown for subgroups of patients in Table 2. There was no significant difference based upon gender. The median level for 26 patients with a normal urinalysis at the time of study was 2 030 Units/ml (88% with an elevated level). Levels of HAA-

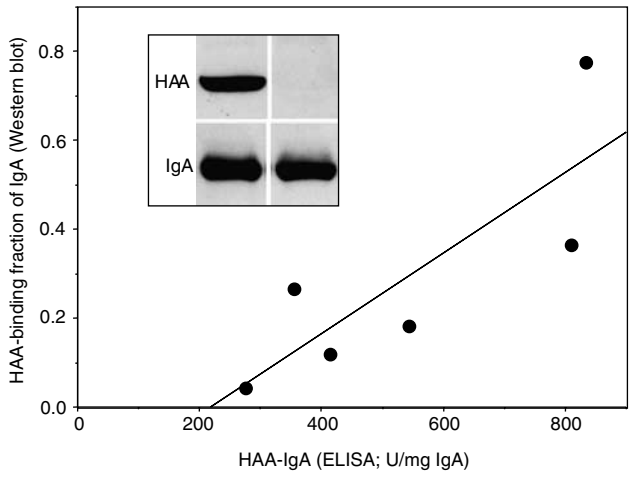

Figure 2 | Reactivity of HAA with serum IgA in ELISA and Western blot assays. ELISA data are expressed as HAA-IgA (U/mg IgA); Western blot data are the ratios of heavy-chain band densities for HAA-binding IgA and total IgA. Six samples with wide range of HAA-IgA reactivities were used. The inset shows the two extremes: a highly reactive sample and a modestly reactive sample.

IgA for subjects with an estimated glomerular filtration rate (GFR) $\geqslant 90$ ml/min/1.73 m^2 were similar to those of patients with an estimated GFR < 30 ml/min/1.73 m^2. Furthermore, the HAA-IgA levels did not significantly differ between patients who had undergone renal biopsy within eight weeks of the blood sampling and patients whose blood was obtained more than five years after biopsy. Serum HAA-IgA levels did not significantly correlate with urinary protein/creatinine (UP/Cr) ratio ($r = -0.128$, $P = 0.211$), estimated GFR ($r = -0.147$, $P = 0.069$), or interval since biopsy ($r = 0.0844$, $P = 0.300$). Thus, the serum HAA-IgA level did not appear to be an indicator of disease activity in our cohort of Caucasian adults with IgAN.

Renal biopsies from 34 patients, who had blood obtained within eight weeks of diagnostic biopsy, were graded according to the Haas system[27] by two investigators (WJC and BAJ) blinded to the clinical and laboratory data. Levels of HAA-IgA were above the cutoff value of healthy controls in five of eight (63%) patients with subclass 1, 2, and 3 histological features and in 20 of 26 (77%) patients with subclass 4 and 5 disease.

DISCUSSION

In this study, we developed a quantitative assay to measure serum Gal-deficient IgA (HAA-IgA). Although the total IgA correlated with the HAA-IgA levels, only the serum HAA-IgA level differentiated IgAN patients from the healthy controls with high sensitivity (Table 2). This finding may be explained by a greater amount of Gal-deficient IgA recognized by naturally occurring anti-glycan antibodies, resulting in formation of CIC.[10]

In our ELISA, serum IgA was captured by a polyclonal IgA heavy chain-specific antibody. As a result, both subclasses, IgA1 that accounts for about 84% of serum IgA[28,29] and

IgA2, are captured. However, only IgA1 has *O*-linked glycans and, therefore, is the only IgA subclass that can bind to HAA, a GalNAc-specific lectin. Consequently, the HAA-IgA assay is a measure of serum Gal-deficient IgA1.

Mesangial IgA deposition is incidentally encountered in asymptomatic individuals in frequencies that vary among studies, ranging from 2 to 16%.[30–34] For example, in a study in Japan, biopsies from 510 newly procured living-donor renal allografts taken at the time of transplantation showed mesangial IgA deposition in 82 (16.1%) of them.[33] By contrast, in a study of necropsy examinations of 756 Finish adults who had committed suicide or met with a violent death, mesangial IgA deposits were found in 18 (2.4%) of the individuals.[32] However, these renal biopsies with IgA deposits rarely showed any significant pathological changes by light microscopy. Consequently, the clinical significance of these observations is not clear, but many otherwise normal healthy individuals may have mesangial deposits of IgA without clinical manifestation of nephritis or pathological changes of the glomeruli. It would have been interesting to have renal biopsy data on our cohort of healthy controls and to assess the frequency of IgA deposits with and without concurrent renal pathology by light microscopy. However, this could not have been carried out for ethical reasons.

Several groups of investigators have shown significantly increased binding of GalNAc-specific lectins to serum IgA1 from patients with IgAN or Henoch–Schönlein purpura nephritis.[10,11,23,35–40] Using HAA, Linossier *et al.*[39] showed increased binding for IgAN patients with normal thickness of the glomerular basement membrane on electron microscopy. Studies with other GalNAc-specific lectins, *Vicia villosa* lectin[23,38] and *Glycine max* lectin,[40] showed increased serum Gal-deficient IgA1 in patients with Henoch–Schönlein purpura nephritis and IgAN, respectively. However, these results did not distinguish the patients from healthy controls; thus, the test would not be adequate to diagnose either disorder.

Several candidates for a serological diagnostic marker of IgAN, such as increased levels of serum IgA or of IgA-containing complexes with fibronectin, IgG, or C3, have been previously examined.[7–9,39–45] Although the serum IgA concentration is significantly elevated in about half of the patients with IgAN,[46,47] this parameter is not satisfactory as a diagnostic test, as confirmed by our study (Table 2). Jennette *et al.*[42] found that 93% of 30 patients with IgAN or Henoch–Schönlein purpura nephritis had serum IgA-fibronectin aggregates, compared with 7% of 30 controls. Later, the same group performed a larger study with 63 adult and 25 pediatric subjects and found IgA-fibronectin aggregates in only 40% of samples.[43] Furthermore, just 48% of the patients ever showed IgA-fibronectin aggregates, despite sampling on more than one occasion.[43] Czerkinsky *et al.*[7] reported the presence of IgA1- and IgG-containing CIC in 40% of 30 patients with IgAN and none in the 14 normal subjects. Coppo *et al.*[8,41] found IgA-CIC in 65% of IgAN patients using a conglutinin-binding assay. The levels of IgA-CIC correlated with clinical activity, as denoted by the magnitude of microscopic hematuria or a history of macroscopic hematuria. Mixed IgA1-IgG CICs were detected in 68% of patients as reported by Schena *et al.*[9] Activated C3 was detected in 30–57% of patients with IgAN.[44,45] To date, none of these assays has been introduced as a diagnostic test.

Using a panel of 15 lectins, our group has examined the reactivity of de-sialylated serum IgA1 from 10 patients with IgAN and 10 healthy controls.[11] All of the GalNAc-specific lectins demonstrated higher reactivity with serum IgA1 from the IgAN patients than with IgA1 from healthy controls; however, the highest specificity was obtained with HAA.[11] The specificity of many lectins (including those specific for hinge-region *O*-linked glycans) may vary by the geographical location of the source (animal or plant) and by the method of preparation. It is thus possible that the HAA from the source we chose had selectivity properties that were superior to those of the other GalNAc-specific lectins we tested.[11,26] These results prompted us to select HAA to use in our lectin ELISA to measure the levels of Gal-deficient IgA1.

Gal-deficient IgA1 likely conveys nephritogenic properties to the IgA1-CIC. Gal-deficient IgA1 has been isolated from the immune deposits in the renal mesangium of patients with IgAN.[17,18] Furthermore, immune complexes with Gal-deficient IgA1 stimulate mesangial cells in culture to proliferate[5,13,15,16,48] and secrete components of the extracellular matrix and cytokines.[49–51] Although the size and biological activity of these CIC may be determined by various factors, including the antigen to antibody ratio,[13] the aberrant glycosylation seems to play a central role in inducing glomerular injury.[52] Thus, measurement of Gal-deficient IgA1 in serum samples may be of diagnostic significance, despite the lack of an association between the serum HAA-IgA level and disease severity, as assessed by histological features, magnitude of proteinuria, or interval since diagnosis. The elevated serum HAA-IgA levels in patients during a clinically quiescent phase many years after clinical onset suggest that defective galactosylation may serve as a biomarker for the disease.

Although Gal-deficiency of IgA1 *O*-linked glycans can be analyzed by Western blotting, gas-liquid chromatography, and mass spectrometry, these procedures are expensive and cumbersome. In contrast, an ELISA is a relatively rapid, inexpensive, and high-throughput assay with excellent reproducibility. We anticipate that the HAA-IgA ELISA could be easily adapted for the clinical laboratory. If the sensitivity of this assay can be improved while maintaining its high specificity, it may be developed into a noninvasive diagnostic test for IgAN.

MATERIALS AND METHODS

Patients and healthy controls

The study included 153 Caucasian patients with IgAN who had undergone biopsy for the evaluation of proteinuria, hematuria, or renal dysfunction. Thirteen patients were younger than age 18 years at the time of biopsy. All patients were older than age 18 at time of

blood sampling for the study. Ninety-three were male, yielding a male to female ratio of 1.68. Blood and urine samples were obtained on one occasion. Mean age at biopsy was 37.0 years (range 10–75 years). The mean interval between the date of diagnosis by biopsy and the date of blood sampling for this study was 4.9 years (range 0–29 years). Thirty-four (22%) patients were studied within eight weeks of biopsy and 47 (31%) had blood drawn more than five years after biopsy. Serum creatinine concentration, urinalysis and random spot UP/Cr ratio were obtained at the time of study. Estimated GFR was calculated using the Modification of Diet in Renal Disease formula.[53] Estimated GFR was $\geqslant 90$ ml/min/1.73 m^2 for 26 (17%) patients, $\geqslant 60$ but <90 ml/min/1.73 m^2 for 40 (26%) patients, $\geqslant 30$ but <60 ml/min/1.73 m^2 for 52 (34%) patients, and <30 ml/min/1.73 m^2 for 35 (23%) patients.

The biopsy-proven diagnosis of IgAN was based upon the demonstration by direct immunofluorescence of IgA as the dominant or co-dominant Ig in a predominantly mesangial distribution and the lack of clinical or serological evidence for systemic lupus erythematosus, vasculitis, or Henoch–Schönlein purpura[3] and the absence of $\geqslant 2+$ mesangial C1q deposition.[54]

The healthy controls consisted of 153 Caucasian residents of Alabama, Kentucky, or Tennessee. All were at least 18 years of age (range 18–80 years). The mean age was 27.0 ± 1.9 years. The group consisted of 74 males and 76 females. Serum creatinine, UP/Cr ratio and blood pressure were measured for each healthy control. Individuals with UP/Cr ratio >0.20 or estimated GFR <60 ml/min/1.73 m^2 were excluded. Estimated GFR was <90 ml/min/1.73 m^2 for 78 (50.9%) of the healthy controls.

The Institutional Review Boards at the University of Alabama at Birmingham and the University of Tennessee Health Sciences Center approved this study. Written informed consent was obtained from all subjects.

Determination of serum IgA and Gal-deficient IgA by ELISA

For measurement of serum total and Gal-deficient IgA, high-adsorption polystyrene 96-microwell plates (Nalge Nunc International, Rochester, NY, USA) were coated overnight with 3 μg/ml F(ab′)$_2$ fragment of goat IgG anti-human IgA (Jackson ImmunoResearch Labs., West Grove, PA, USA) in phosphate-buffered saline. Coated plates were blocked with 1% bovine serum albumin (Sigma Chemical Company, St Louis, MO, USA) in phosphate-buffered saline-0.05%-Tween 20, and serial twofold dilutions of samples and standards in blocking solution (in duplicate) were incubated overnight at room temperature. The standard for IgA consisted of a pool of normal human sera (Binding Site, Birmingham, UK), previously calibrated for Ig isotype concentration by ELISA and radial immunodiffusion. The bound IgA was detected by incubation with biotin-labeled F(ab′)$_2$ fragment of goat IgG anti-human IgA (BioSource, Camarillo, CA, USA) for 3 h at 37°C followed by 1 h incubation with horseradish peroxidase-conjugated ExtrAvidin (Sigma). For quantitation of Gal-deficient IgA, the standard consisted of a polymeric Gal-deficient IgA1 isolated from serum of a patient with IgA1 myeloma. This Ig has been previously shown to mimic the aberrantly glycosylated IgA1 from IgAN patients.[10] To remove terminal sialic acid from *O*-linked GalNAc on IgA, the samples and the IgA1 myeloma protein standard were incubated with 100 μl (1 mU)/well neuraminidase (Roche Diagnostic Corp., Indianapolis, IN, USA) in 0.01 M acetate buffer pH 5 for 3 h at 37°C.[11] After washing, 100 μl biotin-labeled HAA, a GalNAc-specific lectin, (1:500 dilution; Sigma) was added to each well. Following 3 h incubation at 37°C, the plates were washed and horseradish peroxidase-ExtrAvidin (Sigma) was added and incubated for 1 h at 37°C. For both IgA and Gal-deficient IgA, the wells were developed with the peroxidase chromogenic substrate *O*-phenylenediamine-H_2O_2 (Sigma). The color reaction was stopped with 1 M sulfuric acid and the absorbance was measured at 490 nm with an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The amounts of total IgA and Gal-deficient IgA in the tested samples were calculated with the DeltaSoft II program (BioMettalics Inc., Princeton, NJ, USA) by interpolating the optical densities on calibration curves, constructed using serum IgA and a Gal-deficient IgA1 myeloma protein. As the standard IgA1 myeloma protein is not entirely devoid of galactose, the expression of results in μg/ml does not precisely reflect the concentration of Gal-deficient IgA1 in the sera. Therefore, we expressed the results in U/ml. One unit of HAA-IgA was defined as 1 μg of this standard. The results were also normalized to total IgA and expressed in U/mg IgA.

Duplicate serum samples from 21 healthy controls were analyzed to assess the degree of intra-assay variation. The results showed excellent reproducibility ($r=0.970$; $P<0.0001$). To verify the accuracy of the ELISA measurements of total IgA level in serum, five serum samples and the calibration serum were analyzed in the clinical laboratory of the University of Alabama Hospital. The differences in the measurements by ELISA and nephelometry ranged between 2 and 17%, the mean difference was 9 ± 5%.

Western blot analysis

Serum samples from six individuals (four IgAN patients and two controls) representing a wide range of HAA-IgA values were normalized for IgA content (1.0 or 0.1 μg IgA/well for blots to be developed with HAA or anti-IgA, respectively), then reduced with 5% β-mercaptoethanol, and electrophoreticaly separated on a 4–15% gradient sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). The gels were then electroblotted to polyvinylidine difluoride membrane (0.45 μm, Millipore, Billerica, MA, USA). After the transfer, polyvinylidine difluoride membranes were blocked with SuperBlock buffer (Pierce, Rockford, IL, USA) containing 0.05% Tween 20, washed and treated for 3 h at 37°C with 10 mU/ml neuraminidase (Roche). Membranes were then washed and incubated overnight at 4°C with 1:500 dilution biotin-labeled HAA (Sigma) in blocking buffer. To detect the heavy chain of IgA, a duplicate membrane was incubated overnight at 4°C with biotinylated goat F(ab')$_2$ IgG anti-human IgA (Biosource). The biotin-labeled probes bound to IgA were detected, after addition of streptavidin-horseradish peroxidase conjugate, with SuperSignal West Pico Solution (Pierce). The positive bands were visualized using X-ray film (Kodak, Rochester, NY, USA) and the films were analyzed by densitometry to calculate the ratios of HAA-IgA to α-chain.

Statistical analysis

Wilcoxon rank sum test was used to compare subject and control groups for serum concentrations of IgA, HAA-IgA levels, and relative HAA-binding to IgA. The 90th percentile for healthy controls was the cutoff point for calculation of sensitivity and specificity. The receiver operating characteristic curve for HAA-IgA levels in patients and controls was constructed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The Spearman correlation coefficients were used to assess the relationship between HAA-IgA levels and UP/Cr ratio, estimated GFR and the interval since diagnosis by biopsy. SAS 9.1 (SAS Institute, Cary, NC, USA) was used for descriptive statistics and calculation of correlation coefficients.

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